Universidade de Aveiro   Departamento de Biologia

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TESTING MACROEVOLUTIONARY SKULL PATTERNS USING TETRAPOD CRANIAL NETWORKS

TESTANDO PADRÕES MACROEVOLUTIVOS EM TETRÁPODES COM RECURSO A REDES DE OSSOS CRANIANOS

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Aos meus pais por sempre acreditarem em mim

o júri

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resumo   No corpo dos vertebrados o crânio aloja o cérebro e diversos órgãos sensoriais importantes. Esta estrutura anatómica passou por várias modificações e especializações que recapitulam o processo evolutivo. O facto de ser muito variável, complexo e de fácil preservação torna-o uma estrutura comummente utilizada em anatomia comparada e em estudos de biologia evolutiva, nomeadamente para a classificação de vertebrados e reconstruções filogenéticas.

Na análise aqui apresentada consideramos um total de 25 espécies: *Acanthostega gunnari*; 14 espécies dos Mammalia incluindo os grupos Sirenia, Hyracoidea, Carnivora, Cetartiodactyla e Primatas e 10 Reptilia distribuídos entre Crocodylia, Neotheropoda, Squamata, Rhynchocephalia e Testudines.

Todos os dados recolhidos foram analisados em três *softwares* diferentes: PAUP, para uma análise filogenética, Gephi para construir redes de contactos dos ossos do crânio e Rstudio para aferir dados estatísticos.

No presente trabalho mostramos que a filogenia de um animal pode ser inferida até ao nível da classe usando somente dados de contactos cranianos, no entanto, não é suficiente para reconstruir árvores filogenéticas.

keywords   Evolution, phylogeny, skull bone networks, Tetrapoda, vertebrates

abstract

The vertebrates' skull houses the brain and important sensory organs. This anatomic structure has suffered various changes and specializations that recapitulate the evolutionary process. This variability, its complexity and easy preservation makes it one of the standard characteristics used in comparative anatomy and evolutive biology for instance in the classification of vertebrates and phylogenetic reconstruction.

A total of 25 species were considered: Acanthostega gunnari; 14 species within Mammalia including the groups Sirenia, Hyracoidea, Carnivora, Cetartiodactyla and Primates and 10 Reptilia from Crocodylia, Neotheropoda, Rhynchocephalia, Squamata and Testudines.

All the collected data was analyzed through three different softwares: PAUP, for phylogenetic analysis, Gephi to build networks of contacts from the skull and Rstudio for statistics.

In this study we show how phylogeny of an animal can be inferred to Class level using exclusively information regarding skull bone contacts but there is not enough information contained on the skull alone to recreate phylogenetic paths to build complete phylogeny.

Index

Index of figures

Index of tables

Introduction

Through the study of fossilized remains paleontologists can make conjectures about characteristics such as behavior, reproduction and appearance of extinct animals (Alexander 2011).

For a better understanding of the evolutionary sequences at the morphological level it is essential to determine phylogenetic relationships between existing and extinct species over large periods of time (Lovtrup 1977; Minelli 2015).

All vertebrate clades that inhabit land ecosystems are tetrapods, however the origin of this group has been the subject of discussion and there are still multiple hypotheses (Patterson 1980). Lobe-finned fishes and coelacanths are arguably the most probable ancestors of Tetrapoda (Long and Gordon 2004; Schultze and Campbell 1987). Traits that seem to link the first group and tetrapods are thought not to be due common descent but convergent evolution or reversals although it was determined by Meyer and Wilson (1990) a close relationship between these two through partial DNA sequences from two conservative mitochondrial genes.

Donoghue et al. (1989) affirmed that including fossils in cladistic analyses may substantially alter the inferred phylogenetic relationships among extant taxa and the hypothesis on character evolution. They suggest that every effort must be made to incorporate fossil analyses in taxonomic studies.

All paleontological, neontological, and molecular data plus rigorous cladistic methodology should be used to further interpret the sequence of morphological events and innovations associated with the origin of tetrapods when relating fossils and extant taxa in a phylogenetic framework (Meyer and Dolven 1992). That can be problematic since the quality of the fossil remains is not always high enough, there's no biological material and hardly any neontological available data from these long extinct animals which usually leads to differences among paleontologists in the interpretation and lack of a consensus for the branching order among lobe-finned fishes. (Meyer and Dolven 1992)

The first tetrapod fossil evidence come as footprint trails from the Middle Devonian, 390 million years ago (Narkiewicz and Narkiewicz 2015) and the first fossils from Stegocephalians from the Late Devonian dating from 375 million years ago (Losos 2013). These first tetrapods had and amphibian lifestyle and were still dependent of water for their reproduction (Losos 2013). The appearance of the amniotic egg allowed the postures to be made in a dryer environment allowing the embryonic growth to became more independent of the water. This evolutionary novelty led to the domination of other habitats during the late Paleozoic and all of the Mesozoic (Losos 2013). During the Devonian, Carboniferous and Permian the fossil record reveals the appearance of very diverse life forms (flying, crawlers arboreal, fossorial, etc) yet retaining some of the ancestral sarcopterygii features such as bony skulls and their patterns that are still recognizable today (Northcut 1987; Rosen et al. 1981).

The skull is a complex bone structure that holds part of the nervous system and major sensory organs such as ears, mouth, nose and eyes, protecting the brain while providing structural support for muscle attachments. (Anderson et al. 2018)

The need to adapt to very diverse habitats and life styles has led the skull to go through different specializations due to which it has become one of the main features for tracing phylogenetic relationships within Tetrapoda (Gregory and Hellman 1939). There are many cases, particularly in comparative anatomy and paleontology, of taxonomists using only morphological skull characters when describing a new taxon as the skull presents a complexity not present in post-cranial skeleton (Castanhinha 2014). A preliminary review over a sample of 38 paleontological papers describing newly found extinct species revealed that 74% of them use cranial character in their diagnosis (see methods and supplementary materials).

At the same time tendency for skull simplification through the evolution of tretapods either by loss or fusion of bones from their origin in ancestrals to the extant forms is documented as "Williston's Law" and is generally accepted today (Gregory et al. 1935, Williams 1966, Bonner 1988, Hildebrand 1988, McShea 1991, Valentine et al. 1994, Sidor 2001). However, there is unresolved questions as to what is the

cause of this reduction in the number of cranial bones? What is the role of constraints, convergence and contingency in the way the skull has evolved? Do the homologies used by different authors make sense?

Several hypotheses for taxa relationships have been formulated using the skull as it has markers of the evolutionary trends (e.g. the number of openings in the skull for amniote classification) (Novacek 1993; Bhullar et al. 2012) as well as ontogenic data on the reconstruction of phylogeny (Bhullar et al. 2012).

It is well stablished that the skull provides features useful for phylogenetic classification. However, there are other complementary studies focused on soft tissue composition and different timings of development (Hanken and Thorogood 1993). Information regarding soft tissues of extinct animals is very scarce, which limits the fossil paleobiology studies.

There are thousands of new species of fossil vertebrates that have been described and most of them have cranial character in their diagnoses. However, there are very few studies trying to test whether these characteristics are informative to distinguish extant biological species (Mayr 1999). Usually new extant species are described not only by their anatomy but also by adding other complementary characters (genetic, behavioral, ecological, etc.) (Novacek and Wyss 1986). However, it remains to be proved how comparable the estimates of biodiversity indexes are over the geological timescale, since the majority of fossil species are exclusively described based of bony characters on a morphological concept of species.

Among the cranial characters most commonly used to describe new fossil species are the contacts of the skull bones (Anderson and Kharazi 2018). It is assumed that contacts of cranial bones are useful for reconstructing tetrapod phylogeny but little has been done to test this premise. In this work we approach this idea as a hypothesis and present several methods to test it, such as building parsimony phylogenetic trees and bone contact networks.

Network theory in recent analyses demonstrates that character complexity can be quantified more accurately as a function of the relational properties of the system's components than as the number of elements (Sporns 2002; Newman and

Forgacs 2005; Proulx et al. 2005; Newman et al. 2006; Mason and Verwoerd 2007; Dunne et al. 2008; Knight and Pinney 2009). These methods have been recently applied to study anatomical systems and major evolutionary trends, specifically in tetrapds (Esteve-Altava et al. 2011, Esteve-Altava et al. 2013; Rasskin-Gutman 2003).

When representing the skull as a network, bones are coded as nodes and the contacts as links of a network resulting in a simple and easy way to observe connectivity patterns among individual bones, existence of modularity, patterns in loss or fusion of skull bones, detect changes in their structural arrangement and creates an operative framework for the early comparative anatomy. (Esteve-Altava et al. 2013)

That being said, network theory method can hold the potential to provide a relevant new insight into vertebrates' phylogeny. However, the application of such techniques has never been tested and publish from what we have read until now. Here we compare the widely accepted phylogeny between extant and extinct tetrapods to one build over skull bone contacts and analyze networks of skull bone contacts from 2 groups: Reptilia and Mammalia, and a possible common ancestor *Acanthostega gunnari* an early amphibian like vertebrate.

Materials and Methods

Bone homologies

Only homologous traits are relevant in studies that involve comparing structures for constructing phylogeny, so homologies must be clearly defined (Wagner 1989; Castanhinha 2014). The homologies of mammalian skull elements are now fairly well established and rely on works such as comprehensive studies across extinct and extant taxa integrating embryological and paleontological data (Koyabu et al. 2012) (fig. 1 and table 1). We conducted a review on literature that describes skull morphology and development in reptiles, aves and mammals so we could access the different bone homologies and nomenclatures given by different authors (list 1). Based on this review we chose to work with the most consensual homologies generally accepted by the majority of the authors (table 2).
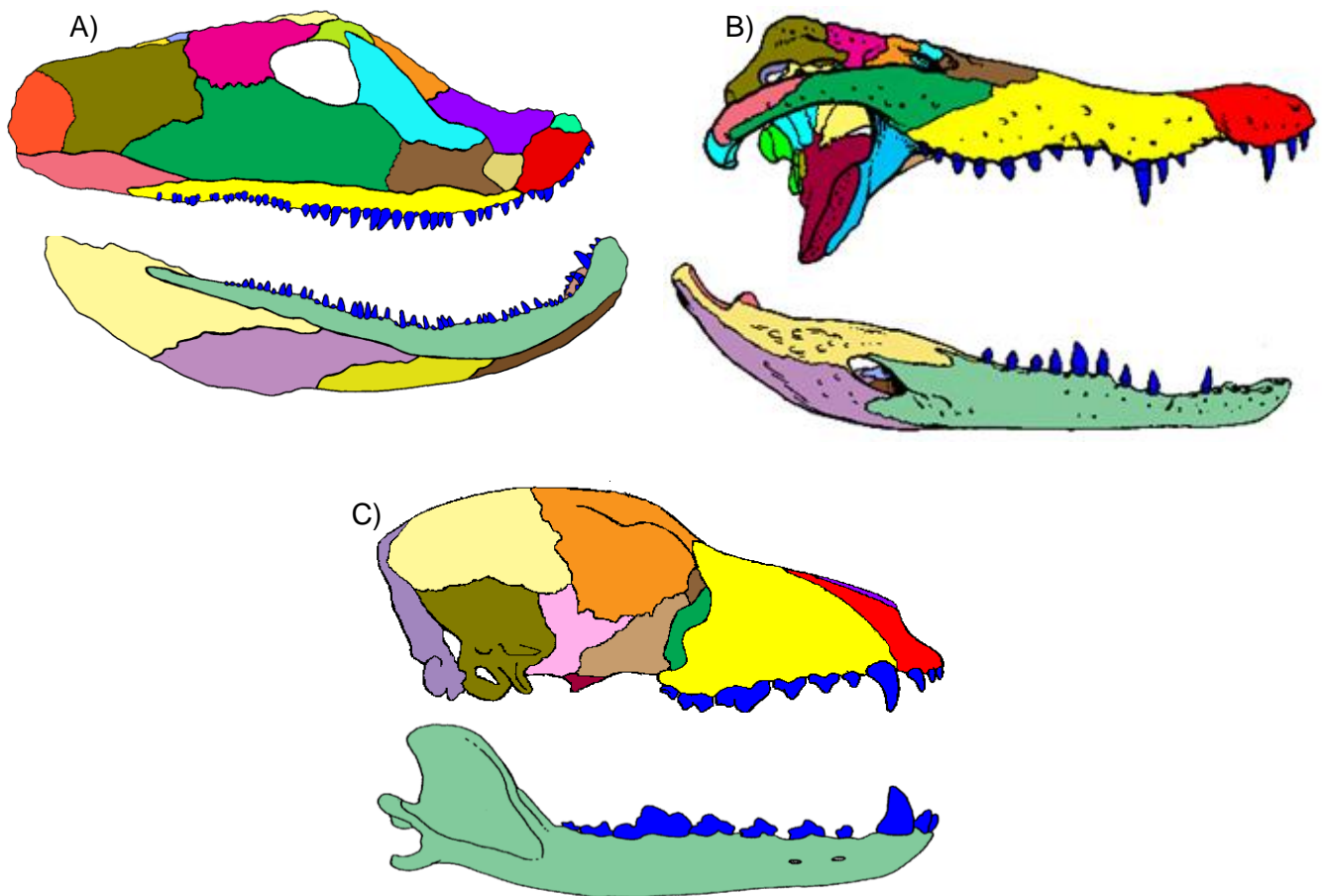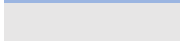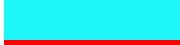
Fig 1. A) *Acanthostega gunnari* skull adapted from Porro et al. 2015; B) Skull from Reptilia specimen *Crocodylus niloticus* adapted from a Chris Brochu image; C) Mammalian skull from *Canis lupus* (adapted from Martin et al. 2011)*.*

Table 1. Predefined skull bone's color code used on fig.1

Bone name	R	G	B	
Bone name	R	G	B	
Adsymphysial	202	147	130	
Alisphenoid/Epipterygoid	150	255	71	
Angular/Ectotympanic	189	140	191	
Anterior tectal	226	212	115	
Arteries				
Articular/Malleus	238	20	91	
Basisphenoid	171	160	0	
Basioccipital	0	255	0	
Brain				
Columella/Stapes	26	150	136	
Coronoid 1	155	181	225	
Coronoid 2	231	230	230	
Coronoid 3	213	86	42	
Dentary	130	202	156	
Ectopterygoid	0	191	243	
Entotympanic/Endotympanic	235	173	61	
Epiotic	255	41	41	
Ethmoid/Mesethmoid	249	113	143	
Exoccipital	161	134	190	
Frontal	248	148	29	
Jugal	0	166	81	
Labyrinth				
Lacrimal	140	98	57	
Laterosphenoid	255	245	104	
Maxilla	255	255	0	
Median rostral	5	248	153	
Nasal	150	0	255	
Nerves (cranial)				
Orbitosphenoid	210	48	37	
Opisthotic/Mastóide	37	113	143	
Palatine	198	156	109	
Palpebral	128	128	128	
Parasphenoid	230	160	122	
Parietal	255	248	153	
Postfrontal	181	230	29	
Postorbital	237	0	140	
Postparietal	195	75	30	
Postsplenial	225	223	21	

Prearticular	36	247	15	
Prefrontal	29	245	248	
Premaxilla	255	0	0	
Preopercular	255	82	40	
Presphenoid	146	174	144	
Prootic	135	129	190	
Pterygoid	158	0	57	
Quadrate/Incus	0	255	255	
Quadratojugal	242	109	125	
Sclerotic ossicles	192	192	192	
Septomaxilla	20	210	17	
Splenial	117	76	36	
Squamosal	130	123	0	
Stylohyal + Tympanohyal	248	8	8	
Supraoccipital	70	21	54	
Supratemporal	153	168	255	
Surangular	250	230	120	
Tabular	255	233	40	
Teeth	0	0	255	
Veins				
Vomer	15	120	57	

List 1. Bones with unambiguous nomenclature.

Adsymphysial	Anterior tectal
Basioccipital	Basisphenoid
Coronoid	Dentary
Epiotic	Exoccipital
Frontal	Jugal
Laterosphenoid	Maxilla
Median rostral	Nasal
Orbitosphenoid	Palatine
Palpebral	Parasphenoid
Parietal	Postfrontal
Postorbital	Postparietal
Postsplenial	Prefrontal
Premaxilla	Preopercular
Preparietal	Presphenoid
Prootic	Pterygoid
Quadratojugal	Sclerotic ossicles
Septomaxilla	Splenial
Squamosal	Supraoccipital
Surangular	Tabular
Teeth	Vomer

Table 2. Homologue bones with different names in Reptilia and Mammalia.

Reptilia	Mammalia	Authors
Alisphenoid	Epipterygoid; Pleurosphenoid	De Beer 1937; Rieppel 1976
Angular	Ectotympanic	Anthwal et al. 2013
Articular	Malleus	Anthwal et al. 2013
Columella	Stapes	Anthwal et al. 2013
Ectopterygoid	Transpalatine	Gregory et al. 1917
Entotympanic	Endotympanic, Metatympanic	Maier 2016
Ethmoid	Mesethmoid	Gregory 1917; Ali et al. 2008
Lacrimal; preorbital	Lacrimal	Witmer 1995; de Beer 1937
Opisthotic	Mastoid; Paroccipital	Gregory 1917
Postparietal	Interparietal	Gregory 1917; Koyabu et al. 2012
Prearticular	Gonial	Gregory 1917; Anthwal et al. 2013
Quadrate	Incus	Gregory 1917
Supratemporal	Suprasquamosal; Supramastoid	Gregory 1917

Collected samples

We studied the skulls from a primitive amphibian, the Reptilia and Mammalia groups since bone homologies between these groups are better resolved, and the phylogeny between and within these groups is generally accepted.

We identified the contacting bones in each skull preferentially by direct observation of the specimens or by searching the literature, alternatively.

Most of the skulls analyzed were observed by more than one person, often two people. When in doubt we discuss the results and it was registered what both observers agreed.

This study includes a total of 25 species. This sampling was made within the species available (either specimens from museums or private collections - see table 8 in supplementary materials for origin of the specimens used and additional bibliography) to represent the disparity present in the considered clades.

We used *Acanthostega gunnari* as a representative of the anatomical ancestor stage to the Mammalia and Reptilia clades. From Mammalia we have studied specimens from Sirenia - *Dugong dugon*, Hyracoidea - *Procavia capensis*, from Carnivora - *Canis lupus*, *Genetta genetta*, *Herpestes ichneumon*, *Panthera leo* and *Vulpes vulpes*, from Certartiodactyla - *Mesoplodon bidens*, *Hippopotamus amphibius*, *Capreolus capreolus* and *Sus scrofa*, and from Primates - *Papio papio*, *Gorilla gorilla* and *Homo sapiens*. In Reptilia we analysed *Alligator mississippiensis*, *Crocodylus niloticus*, *Gavialis gangeticus* and *Osteolaemus tetraspis* from Crocodylia; *Salvator merianae* from Squamata. We also included a skull of *Sphenodon punctatus* from Rhynchocephalia, a sister order to Squamata. We included three Testudines, two *Cryptodira: Dermochelys coriacea* and *Testudo hermanni* and one Pleurodira: *Euraxemys essweini*. Lastly, we chose to include the Aves specimen (*Gallus gallus* from Neotheropoda order) in Reptilia instead of in an isolated group since this work taxonomy is from a Phylogenetic classification system's perspective – which takes in account how species evolve and their direct common ancestors; instead of the Linnaean Classification's view according to which organisms are classified by their unique characteristics regardless of the ancestors they descend or might have descended from.

The collected datasets were organized in bone contact pairwise matrices that worked as basis to an analysis in three different software packages: PAUP to run a phylogenetic parsimony tree, Gephi to construct skull bone networks and Rstudio for statistical analyses (fig.2).

Skull bone contact matrices

We built a symmetrical pairwise matrix containing the Tetrapoda skull bones (Fig. 2). Here we present the steps from the protocol which we used to fill the skull bone contact matrices in this work:

1. Register all references used and catalog numbers of all specimens used to fill each matrix.
2. Register all contacts, contact doubts (e.g. the pair of bones do not contact externally but it is possible that they contact internally).
3. Double check the symmetry of each matrix.
4. If needed localize all cells that are asymmetrically filled and correct all errors.

Criteria:

We used only information from adult specimens. If we had access to subadults, juveniles or embryos, we registered that information in a separate place.

Even if a bone only contacts very marginally with another bone we coded that as a contact. Only absolute and complete absence of contact is coded as an absence of contact.

Contacts between mandibulae and crania are registered only at the mandibular joint level (e.g. articular-quadrate, dentary-squamosal). We assume the mandible is an independent element, thus there are no other contacts between cranial and mandibular bones.

When using illustrations to fill the matrices, some bones may be present inside fenestrae. However, some authors do not illustrate those bones in the background. In such situations, we attributed a doubt to contacts between bones surrounding the fenestrae and to those which we think that might be visible inside the fenestrae in each orthogonal view.

Bone contacts tend to be conserved within each species but there are some exceptions. We used, whenever possible, the most detailed anatomical description made for each species and then analyze personally at least one (preferably more) skulls in museum collections. For example, if we observed 3 skulls with a particular contact and 3 other skulls (from the same species) without that contact, we coded that contact as a doubt.
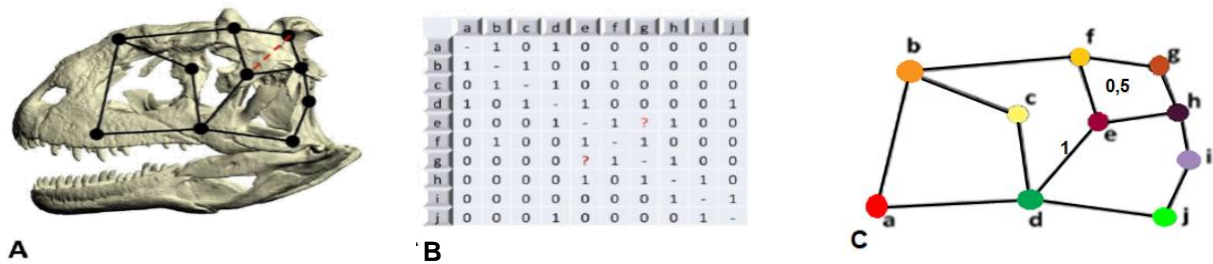
Fig 2. Example of the pairwise bone contact matrix. (A) skull bones marked as vertices (black dots), contacts marked as edges (black lines – observed contacts, dashed red line – dubious contact); (B) symmetrical adjacency matrix with "1" observed contacts between pairs of bones, "0" observed absence of contact and "?" dubious contact (adapted from Sampson and Witmer 2007); (C) Network of bone elements "a" to "j".

Morphing excel matrices into network graphs

After filling the matrices for all the referred species, we used a free access network building software (Gephi) to generate the skull bone networks.

To import the data we firstly went to the excel file and replaced all doubtful contacts with the value of 0.5; all clear contacts 1 and we left empty space for all inexistent contacts; then we copy the values and paste it in a new excel file using the "values only" option. Save as "CSV (separated by commas)".

Open Gephi and on the separator "file" click "import spreadsheet". While importing choose the following options: "Separator: Semicolon"; "Import as: Matrix"; charset: UTF-8 then hit "next", "Intervals" usually appears as default, "conclude", Graph Type: Undirected.

Rstudio

With the bone contact matrices as basis for the absolute number of skull bone contacts of each group we have run a statistics analysis on Rstudio using the coding given on supplementary materials.

PAUP

To run the phylogenetic analysis in order to build the most parsimonious tree we followed the steps given at page 65 at the supplementary materials.

Dubious contacts

When a pair of bones would either not contact externally but it was possible that they would contact internally (e. g. when using illustrations to fill the matrices as some bones may be present inside fenestrae), or when intraspecific variability was observed we considered those contacts as dubious. When in this situation it was attributed a 0.5 weight instead of 1.

To normalize the percentage of dubious contacts in each group we divided the number of dubious contacts (*D*) by the maximum number of possible contacts in each skull. (See tables 5,6 and 7 on Results)

$$\frac{D}{\frac{N^2 - N}{2}}$$

Results

Skull Networks

Acanthostega gunnari displays a higher total of bones (41) an average of 6 contacts per bone and a standard deviation of 2.3 contacts. The absolute maximum number of contacts is 9 on maxilla and pterygoid (fig.3).

Reptilia displays a total of 38 bones with a maximum absolute number of contacts of 8 on the quadrate. The group shows an average of 4 contacts per bone and a standard deviation of 2.6 contacts (fig.4).

Mammalia has the smallest amount of skull bones of 27. The absolute maximum contacts is 8 on the maxilla. The average is 6 contacts per bone and the standard deviation is 3.6 (fig.5).

Regarding normalized values, *Acanthostega gunnari* displays the highest values on the bones maxilla (11.5), pterygoid (11) and squamosal and teeth (both 10.3); Reptilia with maximum values on the quadrate (10), maxilla (8.5) and parietal (8); and Mammalia on maxilla (14.8), frontal (13.1) and alisphenoid (12.4). In all 3 networks the referred bones correspond to those also with maximum absolute values in each group (fig.6).

The pterygoid (9 connections in mammals; 17 in reptiles; 9 in *Acanthostega gunnari*) and prootic (13; 9; 4) seem to be hubs since they show a lot of contacts with surrounding bones but, unlike the prootic, pterygoid looks like it is becoming less relevant as the contacts tend to diminish in more derived skulls as well as the surrounding bones as postfrontal, epipterygoid, preopercular, tabular. (fig. 10)

The quadrate has the most absolute number of contacts in Reptilia but very few in Mammalia (fig. 10). In mammals, the frontal assumes the prefrontal and postfrontal contacts with their surrounding bones (fig. 5) when compared with the reptilian skull pattern (fig.4). Same is true for the angular in the lower jaw and bones such as the surangular, splenial and coronoid are lost in more derived skulls.

The alisphenoid, the orbitosphenoid and the squamosal have a very noticeable increase in contacts from the Reptilia to Mammalia (4 in alisphenoid in *Acanthostega gunnari* and Reptilia to 15 in Mammalia; orbitosphenoid that does not

exist in *Acanthostega gunnari*, to 4 in reptiles to 11 in mammals; and squamosal with 8 in *Acanthostega gunnari* to 15 in Reptilia to 20 in Mammalia).

Acanthostega gunnari displays a higher total of bones on the mandibulae (fig.10) adsymphisial and postsplenial not present in Reptilia plus coronoid, surangular and splenial not present in Mammalia.
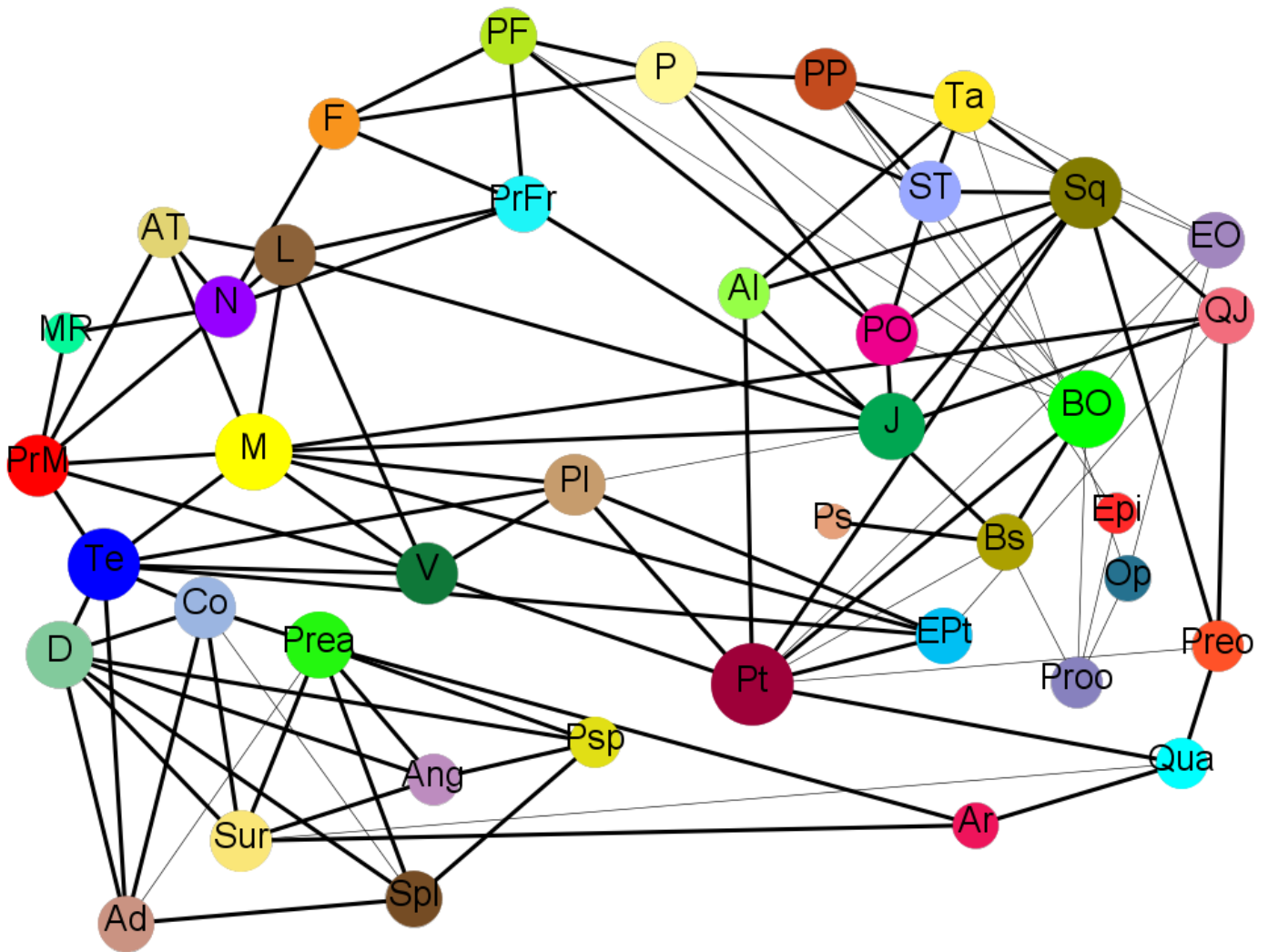
Fig 3. *Acanthostega gunnari* skull network based on bone contacts. Node size is proportional to the absolute number of bone contacts; the thickness of the links quantifies the total of contacts between those two bones.
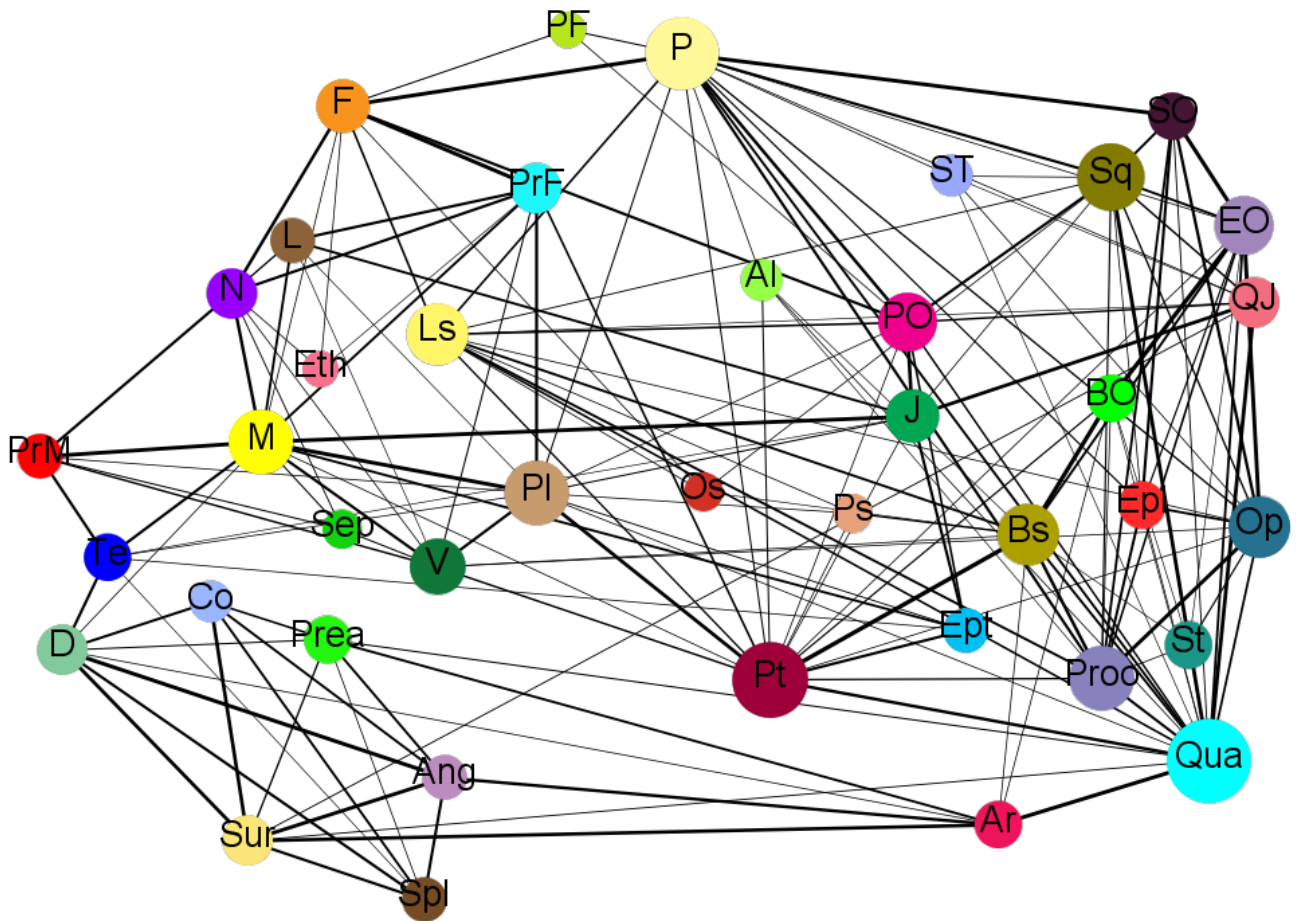
Fig 4. Reptilia skull network based on bone contacts from this group. Nodes' sizes are proportional to the absolute number of bone contacts with the exact same coordinates in all three networks; the thickness of the links quantifies the total of contacts of the species from the group.
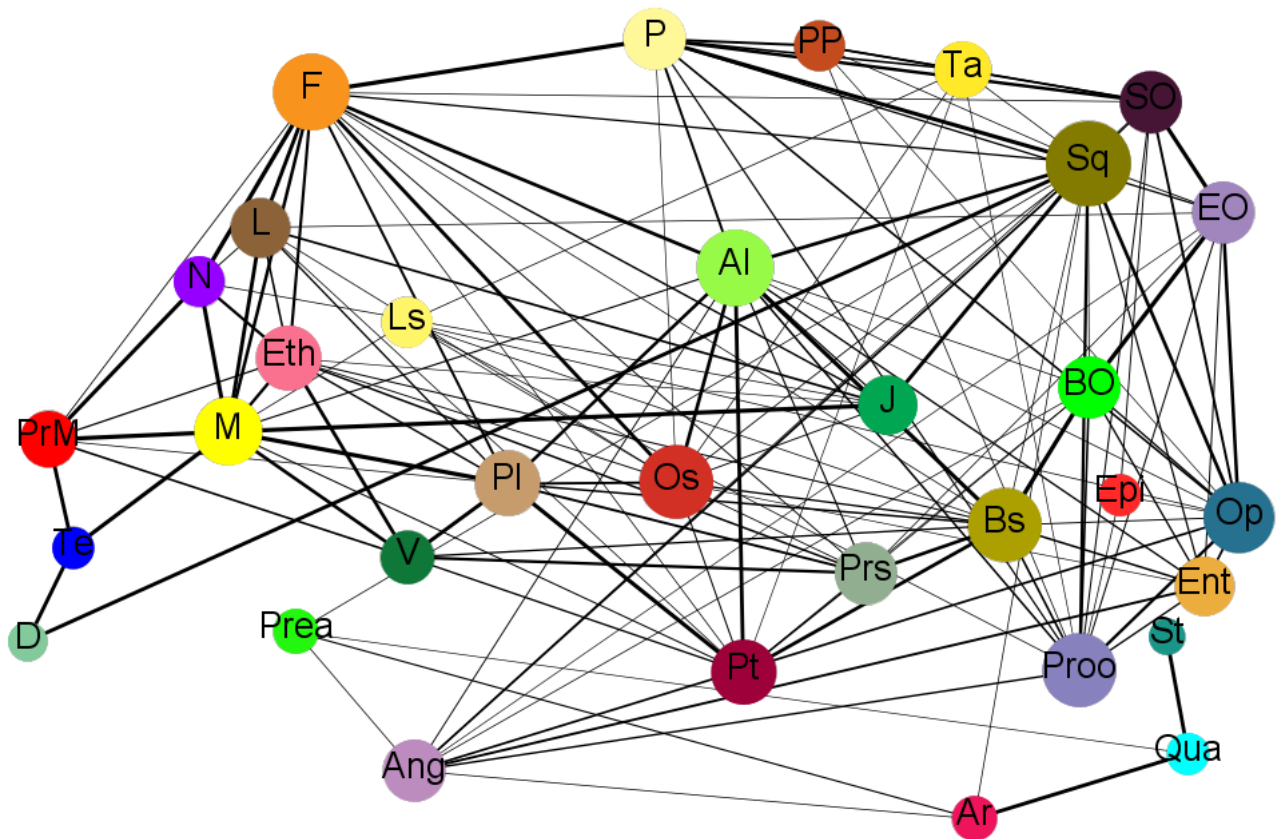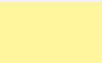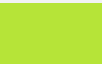
Fig 5. Mammalia skull network based on bone contacts from this group. Nodes' sizes are proportional to the absolute number of bone contacts with the exact same coordinates in all three networks; the thickness of the links quantifies the total of contacts of the species from the group.

Table 3. List of bones, their abbreviations, and respective colors.

Corresponding bone	Label	Color	Palatine	Pl	
Adsymphysial	Ad		Parasphenoid	Ps	
Alisphenoid/Epipterygoid	Al		Parietal	P	
Angular/Ectotympanic	Ang		Postfrontal	PF	
Anterior tectal	AT		Postorbital	PO	
Articular/Malleus	Ar		Postparietal	PP	
Basisphenoid	Bs		Postsplenial	Psp	
Basioccipital	BO		Prearticular	Prea	
Collumela	Cl		Prefrontal	PrF	
Coronoid	Co		Premaxilla	PrM	
Dentary	D		Preopercular	Preo	
Ectopterygoid	EPt		Prootic	Proo	
Epiotic	Epi		Pterygoid	Pt	
Exoccipital	EO		Quadrate/Incus	Qua	
Frontal	F		Quadratojugal	QJ	
Jugal	J		Splenial	Spl	
Lacrimal	L		Squamosal	Sq	
Laterosphenoid	Ls		Supratemporal	St	
Maxilla	M		Surangular	Sur	
Median rostral	MR		Tabular	Ta	
Nasal	N		Teeth	Te	
Opisthotic/Mastoide	Op		Vomer	V	
Orbitosphenoid	Os				

The following chart represents the bone contacts normalized of each bone in each group– *Acanthostega,* Reptilia and Mammalia.
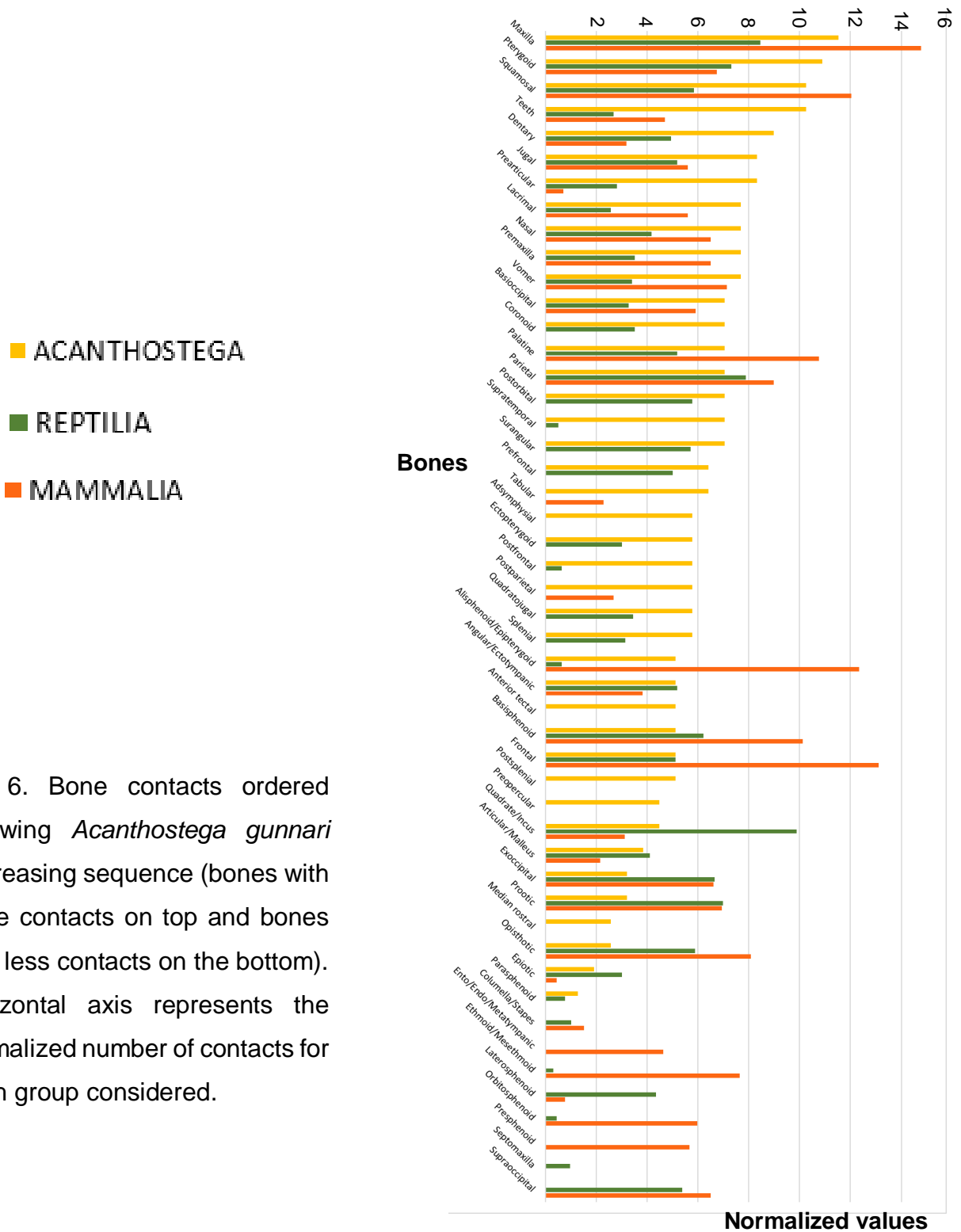
Fig 6. Bone contacts ordered following *Acanthostega gunnari* decreasing sequence (bones with more contacts on top and bones with less contacts on the bottom). Horizontal axis represents the normalized number of contacts for each group considered.

The alisphenoid (6 contacts above average), basisphenoid (4 contacts above average), frontal (7 contacts above average), maxilla (9 contacts above average), palatine (5 contacts above average) stand out in Mammalia as they have a lot more contacts than in the other two groups. Maxilla is the bone with more contacts both in *Acanthostega gunnari* (9) and Mammalia (12) while in Reptilia is the quadrate (13). Then between *Acanthostega gunnari* and Mammalia we have lacrimal (respectively with 8 and 6 contacts), nasal (8 and 7), premaxilla (8 and 7), vomer (8 and 7) and basioccipital (7 and 6) with the most similar number of contacts. The bones with closest results in Reptilia and Mammalia are exoccipital (7), prootic (7), jugal (8 and 6) and pterygoid (7).

It's also important to notice that the quadrate, collumela, prearticular and angular were not previously independent and separate in both Reptilia and *Acanthostega gunnari* contrary to what we see in Mammalia. The quadrate contacts with 10 bones in Reptilia, 5 in *Acanthostega gunnari* and 3 in Mammalia, collumela is not found in *Acanthostega gunnari*, in Reptilia has 10 contacts and 15 in Mammalia; prearticular has 8 contacts in *Acanthostega gunnari* while showing only 3 in Reptilia and 1 in Mammalia, lastly the angular contacts with 5 bones in *Acanthostega gunnari* and Reptilia and 4 in Mammals.

Variability analysis

The bones adsymphysial, anterior tectal, median rostral, postsplenial and preopercular were observed only on the *Acanthostega gunnari*.

The entotympanic, angular and prootic have the most variability on Mammalia (fig.7).

Maxilla, frontal and the alisphenoid from Mammalia have the most contacts but do not display the biggest variability (fig.6 and 7).

In Reptilia the laterosphenoid has the most variability in bone contacts, followed by epiotic (fig.7). Similar to Mammalia, the bones with most contacts are not the bones with higher variability.

It was not possible to infer variability on the *Acanthostega gunnari* bone contacts since we only observed one specimen.
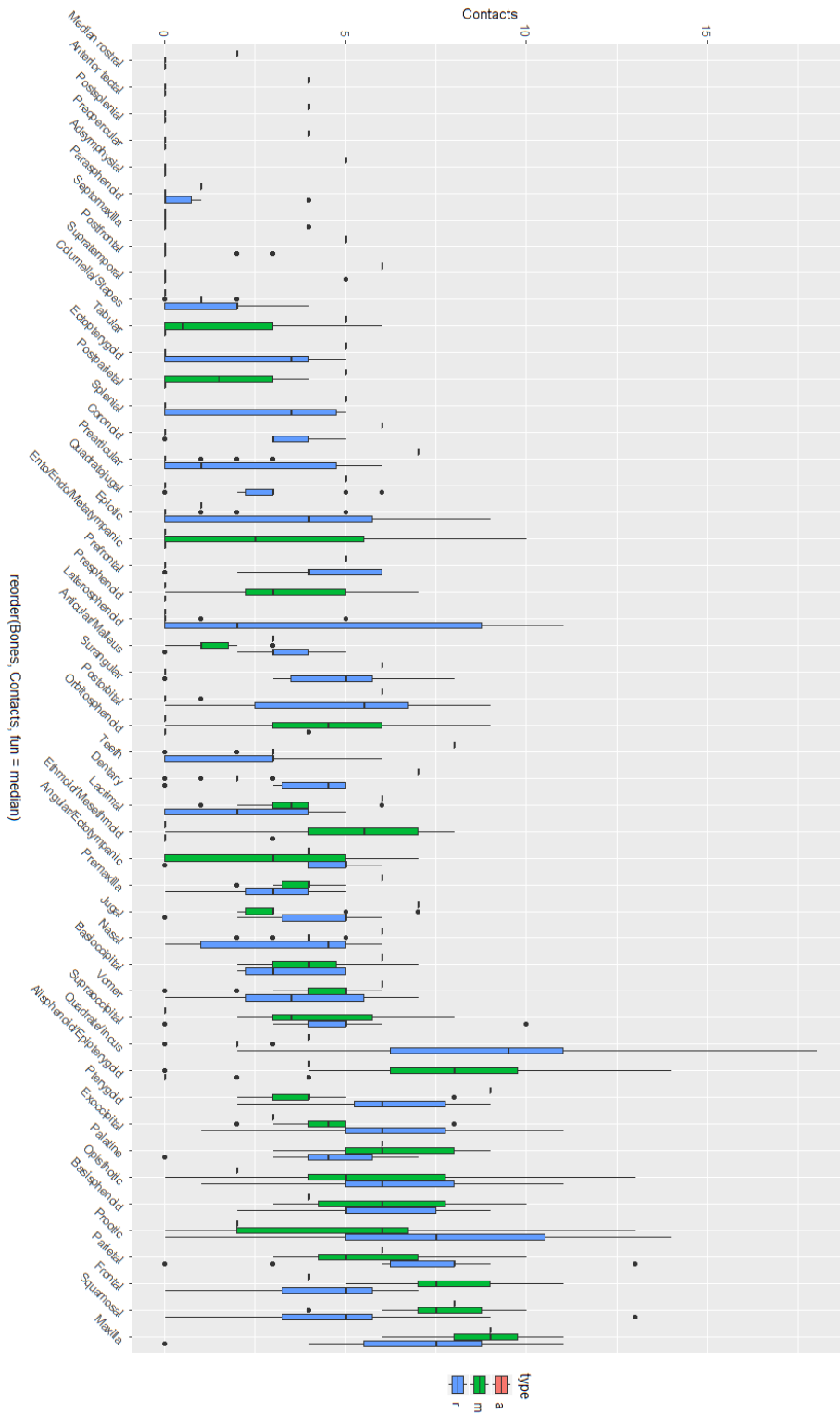
Fig 7. Variability in contacts of each bone in the 3 separate groups ordered by the median of each given bone.

Parsimony tree based on skull bone contacts

We have run a phylogenetic tree using only the species on this study according to the universal accepted phylogeny currently with the help from the website timetree.org to compare the results given by PAUP software. (fig.8)

Using PAUP we imported a bone contact matrix of each species and run a parsimony tree, with one thousand repetitions using *Acanthostega* as an outgroup. (fig.9)
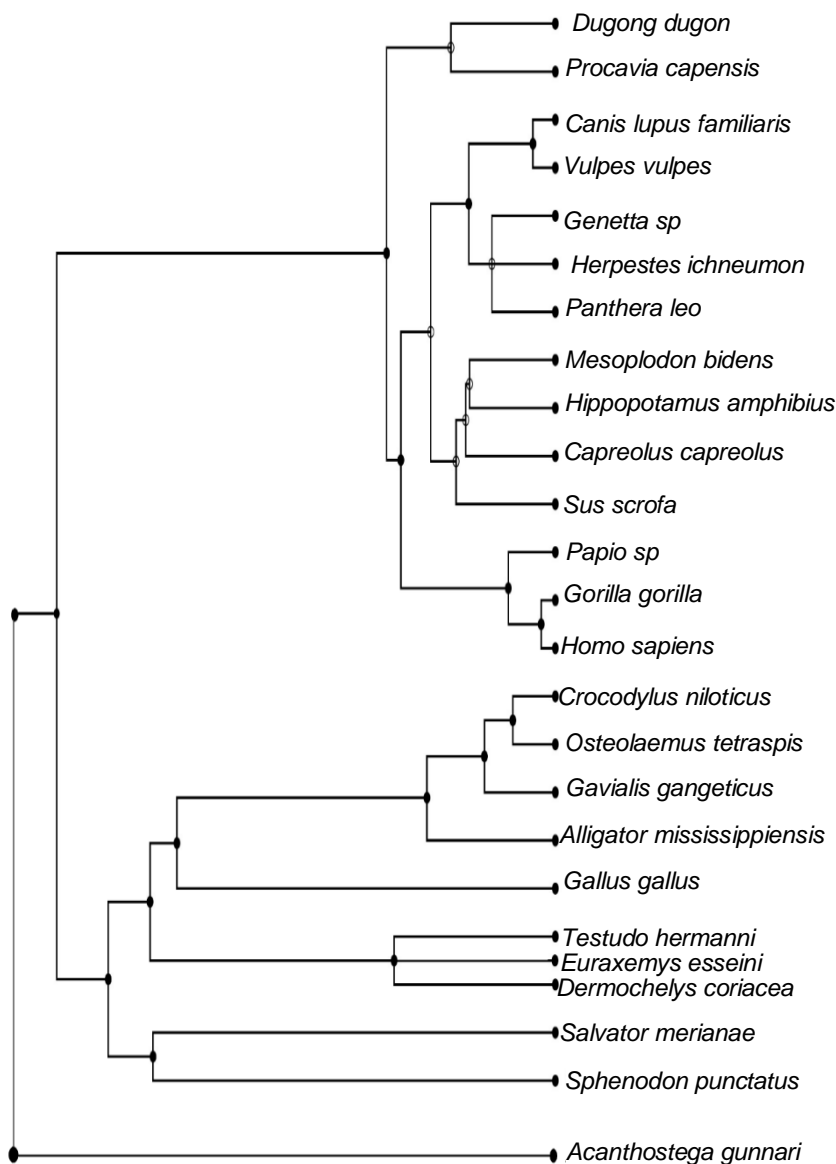
Fig 8. Phylogenetic tree adapted from http://timetree.org/
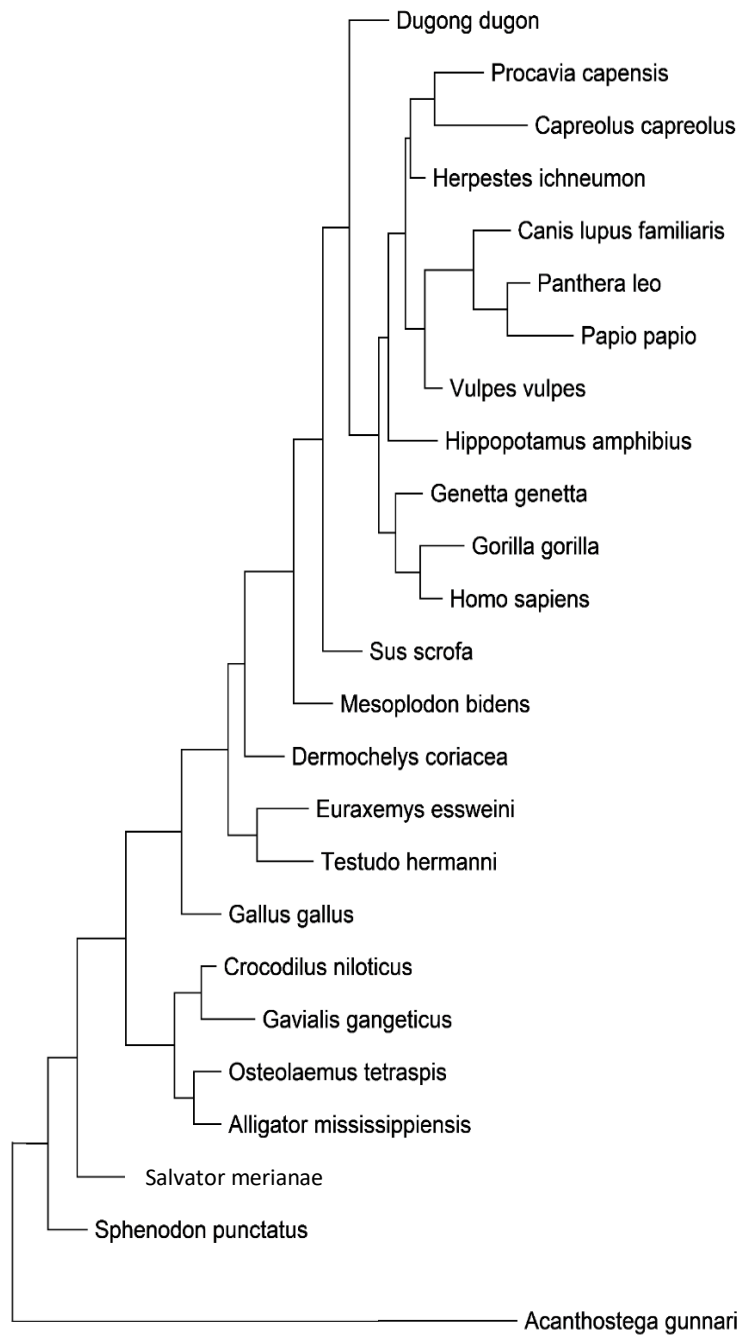
Fig 9. Single most parsimony tree (PAUP software) using bone contact matrix.

Crocodiles are retrieved as monophyletic, as expected; however, the parsimony tree shows inconsistencies with the current acceptable phylogeny on the smaller brackets inside the Crocodylia order (fig.9). For example *Crocodylus niloticus* is known to be in the same clade as *Osteolaemus tetraspis* and not together with *Gavialis gangeticus.*(fig.8)

Sphenodon punctatus is retrieved far apart from all other species (fig.9) which in currently accepted phylogeny is taken as sister group to Squamata and Testudines. (fig.8)

Gallus gallus forms a monophyletic group with the turtles and mammals (fig.9) contrary to what was expected, to be in the same clade as crocodiles since are considered as being from Archosauria. (fig.8)

Turtles on the other side form a paraphyletic group – *Euraxemys essweini* and *Testudo hermanni* belonging in the same Order but apart from the marine specimen *Dermochelys coriacea* grouped with Mammalia. (fig.9)

In Mammalia, *Homo sapiens* and *Gorilla gorilla* show up to be in the same clade but *Papio papio* seems to be misplaced with *Genetta genetta*. (fig.8 and 9)

Cetartiodactyla specimens are all spread out through Mammalia with no defined place (fig. 8 and 9). *Dugong dugon* forms a paraphyletic group with Primates and Carnivores. (fig. 9)

Procavia capensis (an afrotherian like *Mesoplodon bidens* – fig.8) is placed in the same clade as *Capreolus capreolus* (a cetartiodactyla - fig.8) (fig.9) and both in a paraphyletic group with an individual from the Carnivora, *Herpestes ichneumon.*

Vulpes Vulpes is in a paraphyletic group with other carnivores, *Panthera leo* and *Canis lupus*, and a Primate, *Papio papio*. (fig.9)

Dubious contacts

Table 4. Percentage of dubious contacts in Mammalia.

Mammalia	Doubts	Effective contacts	Total	Error % of the whole group	% doubts per specie
Canis lupus	25	87	112		22,3
Capreolus capreolus	6	57	63		9,5
Dugong dugon	8	38	46		17,4
Genetta sp	15	51	66		22,7
Gorilla gorilla	7	58	65		10,8
Herpestes ichneumon	1	52	53		1,9
Hippopotamus amphibius	6	60	66		9,1
Homo sapiens	0	58	58		0
Mesoplodon bidens	0	47	47		0
Panthera leo	13	96	109		11,9
Papio sp	19	95	114	13,2	16,7
Procavia capensis	22	43	65		33,8
Sus scrofa	2	47	49		4,1
Vulpes vulpes	4	56	60		6,7

Table 5. Percentage of dubious contacts in Reptilia.

Reptilia	Doubts	Effective contacts	Total	Error % of the whole group	% doubts per specie
Alligator mississippiensis	9	93	102		9,5
Crocodylus niloticus	15	76	91		16,5
Dermochelys coriacea	2	15	17		11,8
Euraxemys essweini	0	45	45		0
Gallus gallus	3	48	51	15,6	5,9
Gavialis gangeticus	49	92	141		34,8
Osteolaemus tetraspis	15	88	103		14,6
Sphenodon punctatus	11	65	76		14,5
Testudo hermanni	6	55	61		9,8
Salvator merianae	10	52	62		16,1

Table 6. Percentage of dubious contacts in the *Acanthostega gunnari*.

	Doubts	Effective contacts	Total	% Doubts
Acanthostega gunnari	24	111	135	18

Discussion

Acanthostega gunnari represents the primitive Tetrapoda skull by retaining a streamlined head that looks generally like those of the tetrapodomorph fishes (Benton 2014). It displays a higher total of bones (41) but each bone presents a smaller number of contacts (with an average of 6 contacts per bone and 2,3 contacts of standard deviation).

The pterygoid and prootic seem to be hubs since they collect a lot of contacts with surrounding bones but, unlike the prootic, pterygoid looks like it is becoming less relevant as the contacts tend to diminish in more derived skulls as well as the surrounding bones, suggesting that some pterygoid contacts are filled by the prootic.

The quadrate has the largest number of contacts in Reptilia but very few in Mammalia probably due to the fact that this bone becomes part of the middle ear in mammals and also because it is the joint between the lower jaw and skull in Reptilia which is not found in mammals. In *Acanthostega gunnari* there are more bones in the lower jaw (adsymphysial and postsplenial not present in Reptilia plus coronoid, surangular and splenial non-existent in Mammalia and prearticular, angular and articular that migrate to the skull) (fig. 10).

In mammals, the frontal seems to substitute the reptilian prefrontal and postfrontal contacts. Same seems to be true for the angular in the lower jaw as bones such as the surangular, splenial and coronoid are lost in more derived skulls and the angular and prearticular migrate from the mandibulae in Reptilia to the middle ear in Mammalia. The alisphenoid, the orbitosphenoid and the squamosal have a very noticeable increase in contacts from the Reptilia to Mammalia, probably

because there is a reduction in the number of the surrounding bones, however, the epipterygoid maintains the number of contacts on the three groups analyzed as does the vomer and the jugal.

The dentary and teeth become more isolated elements with the loss of most of the bones that would occupy the lower jaw or the migration of the remaining to the middle ear.

In *Acanthostega* and Reptilia, frontal and angular have the exact same number of contacts that can be explained due to the fact that most Reptilia species have very primitive skulls with multiple surrounding bones to the ones that are not present in Mammalia (Goodrich 1916).

The bones with closest results in Reptilia and Mammalia are exoccipital and prootic that we think can be explained since there is not a reduction in the surrounding bones that directly contact with these, columella once it has the same function in both groups it is not unexpected, jugal and pterygoid that are also functionally and positionally very similar in both groups (Romer 1966).

Bones with more contacts in *Acanthostega* are usually those with a higher number of contacts on both Reptilia and mammals.

Our results meet the conclusions from Girgis and Pritchard (1958); Mabbutt and Kokich (1979); Hall (2005) where they find that the loss of bones is followed by the reoccupation by other bones of the space left open giving the possibility of new connections and leads to a reduction in the bone number and increasing the density of connections.

Gregory (1934) concludes that a greater complexity of individual bones compensates for the reduction in number, process that generates more specialized, different anatomical elements, as a result of this reduction in number. According to Aldridge et al. (2002) and Richtsmeier et al. (2006) these results clearly stress the relationship between fusion events during development and the evolutionary trend in skull bone number reduction. Esteve-Altava et al. (2011) argues that there is an emergence of differentiated and more specialized bones when fusions between these are observed and the relative amount of unpaired bones increase as there is

a reduction in effective bone number, that not all skull bones are equally important in maintaining the structure of a skull and thus the stability of the skull against environmental or inherited bone losses can vary according to level of connectivity of the disappearing bones.

It is also important to note that highly connected bones might have a primary role in shaping skull architecture. Some bones (hubs) seem to prevent the collapse of the whole network (skull). It is suggested in Sidor (2001) that bone reduction is phylogenetically found in synapsids, interpreting that simplified, more compact skulls are selectively advantageous. However, this hypothesis is far from being empirically tested and our results cannot help to solve this problem. At the same time, developmental constraints that may favor the loss and fusion of bones or prevent the formation of new ossification centers can play a key role in shaping such evolutionary trends. Riedl (1978) states that losses of less connected bones are responsible for the evolutionary trend in skull complexity emphasizing the direct relationship between connectivity of bones and their structural importance, an idea later supported by Esteve-Altava et al. (2013) adding that there is also a relationship between structural robustness and connectivity and the evolutionary trend in skull morphological complexity. Therefore, the way in which the connections of the skull are reorganized after losses (or fusions) may be the origin of such patterns.

When it comes to phylogenetic analysis, crocodiles are retrieved as monophyletic on the parsimony tree, the results are inconsistent with the current acceptable phylogeny regarding each particular species considered.

Both *Salvator merianae* and *Sphenodon punctatus* are far apart from the others in the obtained phylogenetic tree and this can be explained because the squamate skull is derived from a primitive condition with two openings in the temporal region increasing mobility of the quadrate bone which supports the lower jaw (Goodrich 1966). This diapsid condition defines the subclass Lepidosauria, which includes both Rhynchocephalia and Squamata (Withers and O'Shea 1993) and this may generate homoplastic bone contacts.). However, it was not possible to include Ophidia specimens. It would be interesting to include some Ophidia species

because they are known to have very particular skull shape and articulations (hyperkinetic skull) (Evans 2008).

All reptiles and birds share many characteristics such as only one middle ear bone (Anthwal et al. 2013), sclerotic ring ossicles surrounding the eye with adult bird skulls resembling the juvenile forms of their theropod dinosaur ancestors (Bhullar et al. 2012). Given that, it would be expected that birds should group with crocodilians since they belong to Archosauria (Witmer 1995; Bhullar et al.). Instead, birds unexpectedly form one monophyletic group with mammals and Testudines. This might be explained because of their skull's highly developed specialization and/or due to the presence of homoplasy. However, more sampling from birds is needed to reach any sound conclusion.

Despite strong genomic evidence indicating that turtles evolved from diapsid radiation (which includes all other living reptiles) evidence of such transformation from a single opening ancestral to the anapsid condition of modern specimens remains elusive (Gaffney et al. 2006). The absence of the temporal bone may reflect conservation of the ancestral amniote condition, if so turtles are an extant remnant of an early Reptilia radiation that excludes the other living forms (tuatara, lizards, snakes, crocodilians, birds) (Bever et al. 2015). However, our results show turtles forming a monophyletic group with mammals (with birds as the sister group).

Although the calculated percentage of dubious contacts was high for all three groups (18% for *Acanthostega gunnari*, 15.6% for Reptilia and 13.2% for Mammalia), they are all in a 5% interval, meaning the results and values can be compared.

Conclusions
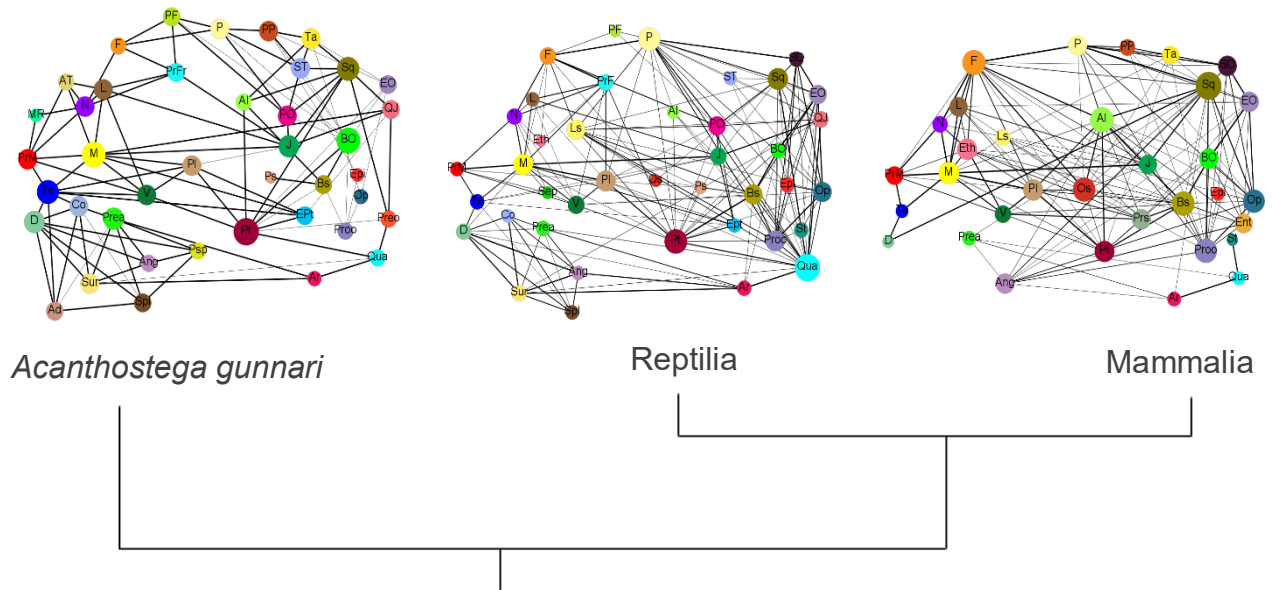
Fig 10. Skull bone networks from the three groups considered.

Primitive skulls tend to have higher absolute number of bones (*Acanthostega gunnari; Reptilia*). *Acanthostega gunnari* has the lowest standard deviation value (2.3). The skull from Mammalia is the most modular as it displays the highest standard deviation value (3.6) and the lowest number of skull bones.

At the Class level, if the specimen is either from Mammalia or Reptilia, the phylogeny can be accurately inferred by analyzing exclusively the contacts of the skull bones.

However, at a less inclusive levels (e.g. order, family, genus and species), bone contacts seem to be homoplastic and phylogeny is not correctly inferred and hence should not be used to diagnose new species without any further empirical data supporting.

The percentage of dubious contacts is similar (<5%) between Mammalia, Reptilia and *Acanthostega gunnari* what can assure that our results are comparable.

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Supplementary materials

Skull characters used to describe new species

We've researched on google scholar and sci-hub scientific work describing a new species. After randomly pick the first ones popping up, resulting on the following table:

Table 7.

Scientific work	Does it use skull characteristics to ID the species?
Wang, Min, Thomas A. Stidham, and Zhonghe Zhou. "A new clade of basal Early Cretaceous pygostylian birds and developmental plasticity of the avian shoulder girdle." Proceedings of the National Academy of Sciences 115, no. 42 (2018): 10708-10713.	yes
Brusatte, Steve. "The rise and fall of the Dinosaurs. *A New History of a Lost World* (2018)	yes
Jessie Atterholt, J. Howard Hutchison, Jingmai K. O'Connor. The most complete enantiornithine from North America and a phylogenetic analysis of the Avisauridae. *PeerJ*, 2018; 6: e5910	no
Greshko, Michael. "It's official: Stunning fossil is a new Dinosaur species". *National Geographic.* 2017	no
Ansuya Bhandari, Richard F. Kay, Blythe A. Williams, Brahma Nand Tiwari, Sunil Bajpai, Tobin Hieronymus. First record of the Miocene hominoid Sivapithecus from	yes

Kutch, Gujarat state, western India. PLOS ONE, 2018; 13 (11): e0206314	
Víctor Fondevilla, Fabio Marco Dalla Vecchia, Rodrigo Gaete, Àngel Galobart, Blanca Moncunill-Solé, Meike Köhler. Ontogeny and taxonomy of the hadrosaur (Dinosauria, Ornithopoda) remains from Basturs Poble bonebed (late early Maastrichtian, Tremp Syncline, Spain). PLOS ONE, 2018; 13 (10): e0206287	yes
Martin Kundrát, John Nudds, Benjamin P. Kear, Junchang Lü, Per Ahlberg. The first specimen of Archaeopteryx from the Upper Jurassic Mörnsheim Formation of Germany. Historical Biology, 2018; 31 (1): 3	yes
Michael J. Ryan, David C. Evans, Kieran M. Shepherd, Hans Sues. A new ceratopsid from the Foremost Formation (middle Campanian) of Alberta. Canadian Journal of Earth Sciences, 2012; 49 (10): 1251	yes
Konishi, Takuya. "Redescription of UALVP 40, an unusual specimen of Chasmosaurus Lambe, 1914 (Ceratopsidae: Chasmosaurinae) bearing long postorbital horns, and its implications for ontogeny and alpha taxonomy of the genus." Canadian	yes

Journal of Earth Sciences 52, no. 8 (2015): 608-619.	
Konishi, Takuya, Michael W. Caldwell, Tomohiro Nishimura, Kazuhiko Sakurai, and Kyo Tanoue. "A new halisaurine mosasaur (Squamata: Halisaurinae) from Japan: the first record in the western Pacific realm and the first documented insights into binocular vision in mosasaurs." Journal of Systematic Palaeontology 14, no. 10 (2016): 809-839	yes
Konishi, Takuya, Paulina Jiménez-Huidobro, and Michael W. Caldwell. "The Smallest-Known Neonate Individual of Tylosaurus (Mosasauridae, Tylosaurinae) Sheds New Light on the Tylosaurine Rostrum and Heterochrony." Journal of Vertebrate Paleontology (2018): 1-11.	yes
Jiménez-Huidobro, Paulina, Michael W. Caldwell, Ilaria Paparella, and Timon S. Bullard. "A new species of tylosaurine mosasaur from the upper Campanian Bearpaw Formation of Saskatchewan, Canada." Journal of Systematic Palaeontology(2018): 1-16.	yes
Adam D. Marsh, Timothy B. Rowe. Anatomy and systematics of the	no

sauropodomorph Sarahsaurus aurifontanalis from the Early Jurassic Kayenta Formation. PLOS ONE, 2018; 13 (10): e0204007	
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We can see that in 38, only 9 do not use skull characters to infer the specie, mostly because it is not present in the remains.

Specimens observed for skull bone contacts matrices build

Table 8.

Species name	Number of specimens used (n)
Acanthostega gunnari	n = 2
Alligator mississippiensis	n = 2
Capreolus capreolus	n = 3
Canis lupus	n = 2
Crocodylus niloticus	n = 5
Dermochelys coriacea	n = 2
Dugong dugong	n = 1
Emys europaea	n = 2
Euraxemys essweyni	n = 1
Gallus gallus	n = 2
Gaviallis gangeticus	n = 4
Genetta genetta	n = 1
Gorilla gorilla	n = 1
Hippopotamus amphibius	n = 2
Homo sapiens	n = 1
Mesoplodon bidens	n = 1
Osteolaemus tetraspis	n = 4
Panthera leo	n = 1
Papio papio	n = 3
Procavia capensis	n = 1
Sphenodon punctatus	n = 3
Sus scrofa	n = 1
Testudo hermanni	n = 1
Salvator merianae	n = 1
Vulpes vulpes	n = 1

Morphing excel matrices into network graphs

1. Open excel sum file and in each page replace 15 for 0.5; 11 with 1 and – for a blank space

2. Copy values from the worksheet with the total sum

3. Paste it in a new excel file "values only"

4. Save as "CSV (Separado por vírgulas)"

5. Open Gephi > file > import spreadsheet

6. While importing choose:"Separator: Semicolon"; "Import as: Matrix"; charset: UTF-8
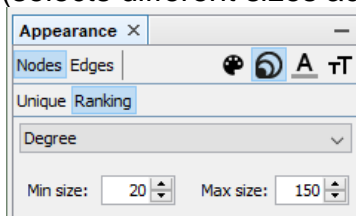
>>Próximo>>

Intervals

>>Concluir>>

Graph Type: Undirected

7.At the workplace:

7.1. On overview, have these options selected

7.1.2.   "Appearance";   Nodes;   size;   Ranking;   Degree (selects different sizes according to their weight (total sum of all contacts))

 Apply.

7.2. At the "Data Laboratory"

7.2.1 Click on the bone's name at the "Id" column, right click on the mouse; edit node; color - click at the three dot square; go to the RGB window – use the color code.

7.2.1.1 same window, write the coordinates give on the excel file.

7.2.2 Label color change "null" to black

7.3. Preview window go to edges; show labels and rescale weight; deactivate "curved"; thickness 10

8.Refresh

(guardar definições. Na matriz seguinte basta escolher as definiçoes guardadas e fazer refresh)

9.Export > SVG/PDF/PNG file
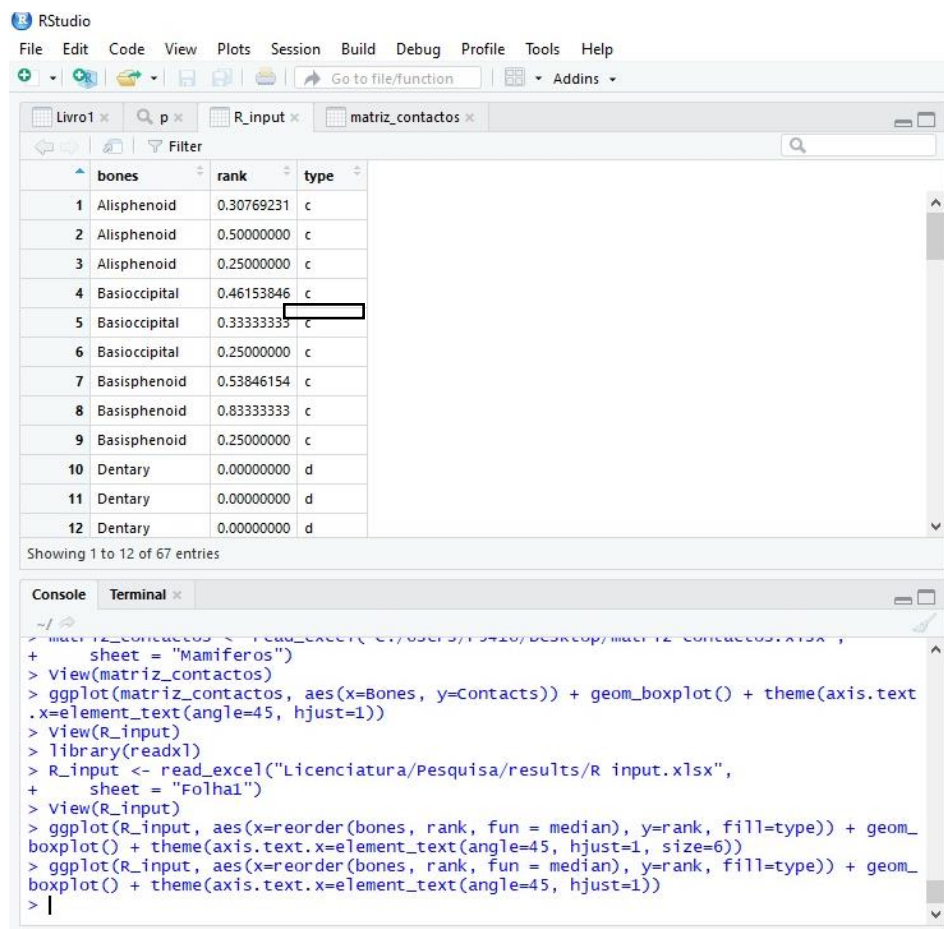
Steps to follow at PAUP (Phylogenetic Analysis Using Parsimony**)**

1. File; Open; Select file; Ok – the first line on the imported file will act as the outgroup
2. Analysis; Parsimony; Heuristic search; Enter
3. Stepwise addition; random
4. 1000 reps, Ok
5. Trees; Show trees; Show
6. Trees; Print/view trees; Save tree to metafile

RStudio protocol

1. Import dataset; From excel; Browse; Import
2. On the "Console" separator paste the following code:

   ```
   >library(ggplot2)
   >ggplot(matriz_contactos,        aes(x=reorder(Bones,      Contacts,
   fun=median),  y=Contacts,  fill=type))  +  geom_boxplot()  +  theme
   (axis.text.x=element_text(angle=45, hjust=1))
   ```


3. Export; Save as image; Width: 1500; Directory
4. Save